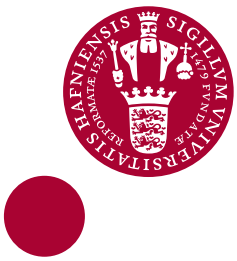

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Published in:
Trends in Plant Science

DOI:
10.1016/j.tplants.2020.03.003

Publication date:
2020

Document version
Publisher's PDF, also known as Version of record

Trends in Plant Science

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Review

Chloroplast Transition Metal Regulation for Efficient Photosynthesis

Sidsel Birkelund Schmidt,[1] Marion Eisenhut,[2,*] and Anja Schneider[3,*]

Plants require sunlight, water, CO_2, and essential nutrients to drive photosynthesis and fulfill their life cycle. The photosynthetic apparatus resides in chloroplasts and fundamentally relies on transition metals as catalysts and cofactors. Accordingly, chloroplasts are particularly rich in iron (Fe), manganese (Mn), and copper (Cu). Owing to their redox properties, those metals need to be carefully balanced within the cell. However, the regulation of transition metal homeostasis in chloroplasts is poorly understood. With the availability of the arabidopsis genome information and membrane protein databases, a wider catalogue for searching chloroplast metal transporters has considerably advanced the study of transition metal regulation. This review provides an updated overview of the chloroplast transition metal requirements and the transporters involved for efficient photosynthesis in higher plants.

Highlights

The transition metals Fe, Mn, and Cu have fundamental functions in the photosynthetic machinery but are also involved in undesired oxidative reactions when in excess. Thus, chloroplast transition metal concentrations need tight regulation to maintain efficient photosynthesis.

Chloroplast transition metal homeostasis is complex. It involves a sophisticated interplay of specific transport proteins, chaperones and carriers, and protein translocation systems.

The availability of full genomic information and organelle-specific protein databases of model plants, such as *A. thaliana*, allows the identification of chloroplast metal transport candidates. Using this information, the two transport proteins CMT1 and PAM71 were recently discovered to function in tandem for delivery of Mn to the oxygen-evolving complex in PSII.

Transition Metal Transporters Are Central to Chloroplast Metal Regulation and Photosynthesis

Chloroplasts harbor three types of membranes: a double (inner and outer) envelope and a thylakoid membrane. The envelope and thylakoid are separated by the stroma compartment. The membranes, and the transporters embedded therein, are selective barriers enabling exchange of ions and metabolites between the cytosol, the stroma, and the thylakoid lumen compartment. Chloroplast functioning and efficient photosynthesis for plant growth rely on the metal cofactor-mediated electron transport chain, in particular the transition metals Fe, Mn, and Cu [1]. However, as ions, their chemical properties can lead to generation of undesired reactive oxygen species (ROS) [2], which, together with their different binding affinities to proteins [3], greatly challenge the use of metals in oxygenic photosynthesis. To avoid harmful generation of ROS, plants chelate metal ions by organic ligands, such as nicotianamine, phenolic compounds, or organic acids (e.g., citrate, malate, ascorbate) [4]. Thus, the transport, homeostasis, and regulation of the individual transition metals are fundamental to optimizing chloroplast functioning.

The chloroplast contains more than 3000 proteins of which about 90 transporters are associated with the chloroplast envelope membranes, controlling the exchange of ions and metabolites [5,6]. However, most of the proteins involved in metal transport activities across the chloroplast membranes remain largely unknown. With the availability of arabidopsis (*Arabidopsis thaliana*) genome information [7] and establishment of various total cellular and more specific chloroplast protein membrane databases [8,9], a wider catalogue for searching new chloroplast metal transporters is now available. Moving forward, reverse genetic studies combined with targeted proteomics and bioinformatics approaches are likely to identify novel transporters [10,11]. This review provides a comprehensive description of chloroplast transition metal requirements, including an update on identified metal transport proteins for the distribution of Fe, Mn, and Cu within the chloroplast. The dynamics and mechanisms allowing the adaptability of the photosynthetic machinery to constantly changing metal concentrations are discussed.

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Chloroplast Transition Metal Requirements

Chloroplasts are rich in transition metals, with up to 80% of total leaf Fe and about 30% of leaf Cu allocated to the chloroplasts [12,13]. In contrast, intracellular Mn is mostly stored in vacuoles and, to a lesser extent, in chloroplasts [14]. The chloroplast metal concentration for Fe, Mn, and Cu is roughly 7 to 10 μg per 10^9 chloroplasts isolated from arabidopsis mesophyll cells, in a ratio of 5.8:1.0:0.5 (Fe:Mn:Cu) [15,16]. Within the chloroplasts, 60 to 80% of Fe, Mn, and Cu is found in thylakoids, reflecting the essential use of these metals in photosynthetic proteins and complexes [12,17], with a total number of 22 Fe atoms, four Mn atoms, and one Cu atom required per photosynthetic electron transport chain (Table 1).

Fe

Fe is the prominent transition metal for protein complexes in the photosynthetic electron chain (Table 1). It is present as Fe^{2+} ligated to PsbA and PsbD in photosystem II (PSII), in heme proteins of PSII and cytochrome b_6/f (Cyt b_6/f), and in Fe–Sulfur [Fe–S] clusters of Cyt b_6/f, photosystem I (PSI), and ferredoxin (Fd) (Figure 1, Key Figure, and Table 1). Biomass and seed yields in arabidopsis can be significantly increased through improved photosynthesis by overexpression

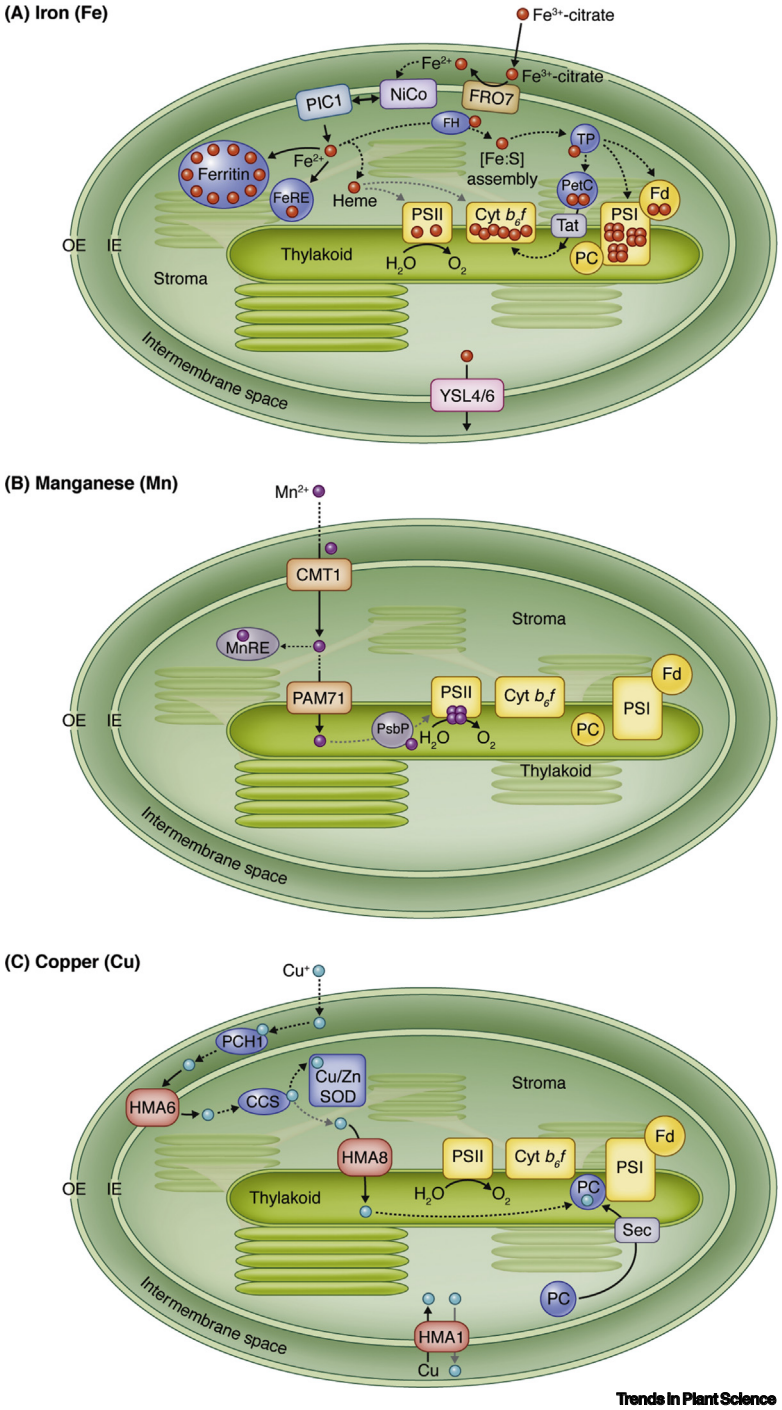
Table 1. Transition Metal Quota in Proteins of the Photosynthetic Electron Transport Chain

Metal	Chloroplast protein (Gene ID)	Ligand/cofactor	Function	Protein Data Bank in Europe	Refs
Mn	PSII PsbA and PsbC (AtCg00020 and AtCg00280)	Mn_4CaO_5	Water oxidation Electron transport	3jcu (spinach), 5xnm, 5xnl (pea)	[103] [104]
Fe	PSII PsbA and PsbD (AtCg0020 and AtCg00270) PsbE and PsbF[a] (AtCg00580 and AtCg00570)	Fe^{2+} heme	Electron transport[e] Photoprotection	5mdx (arabidopsis), 3jcu (spinach), 5xnm, 5xnl (pea)	[105] [103] [104]
Fe	Cyt b_6/f PetB (AtCg00720)[b] PetA (AtCg00540)[c] PetC (At4g03280)[d]	3 × heme heme [2Fe–2S]	Proton translocation Electron transport Electron transport	1q90 (*Chlamydomonas*), 4h44 (*Nostoc*)	[106] [107]
Fe	PSI PsaA and PsaB (AtCg00350 and AtCg00340) PsaC (AtCg01060)	1 × [4Fe–4S] 2 × [4Fe–4S]	Electron transport Electron transport	5zji (maize), 2wse (various plants), 5l8r (pea)	[108] [109] [110]
Fe	Fd Fd1 (At1g10960) Fd2 (At1g60950) Fd3 (At2g27510) Fd4 (At5g10000)	[2Fe–2S]	Electron transport	1pfd (parsley), 1gaq (maize)	[111] [112]
Cu	PC Pete1 (At1g76100) Pete2 (At1g20340)	Cu^+/Cu^{2+}	Electron transport	9pcy (bean)	[113]

[a]Heme bound to PsbE and PsbF constitutes Cyt b_{556} a and b.
[b]Heme bound to PetB constitutes Cyt b_6.
[c]Heme bound to PetA constitutes Cyt *f*.
[d][2Fe–2S] bound to PetC constitutes the Rieske FeS protein.
[e]Fe^{2+} is involved in electron transport, despite no changes in its oxidation state.

Key Figure

Chloroplast Transition Metal Transport



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of the Rieske [2Fe–2S] protein (PetC) [18], implying that Fe availability is not a bottleneck [18]. [Fe–S] clusters have an essential role in linear electron transport but also serve as redox cofactor of the NADH dehydrogenase-like complex (NDH) in cyclic electron flow around PSI [19], a mechanism to balance the levels of ATP and NADPH necessary for efficient photosynthesis. Fe also plays an essential auxiliary function as di-Fe cofactor in the enzyme plastid terminal oxidase (PTOX) [20,21], which is localized to the nonappressed regions of the thylakoids. PTOX protects the plastoquinone pool from overreduction by reducing the number of electrons available for photosynthetic reactions, specifically under abiotic stress [20,21].

In addition to the crucial role in photosynthesis, Fe is also involved as cofactor in many stroma and envelope localized processes. In these compartments, Fe-containing superoxide dismutase (FeSOD) [22] catalyzes the dismutation of harmful superoxide radicals ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2), which is further decomposed by heme-containing ascorbate peroxidases (APX) to produce H_2O and O_2 [23]. Moreover, [Fe–S] clusters occur as cofactors of enzymes involved in chlorophyll metabolism, including chlorophyll *a* oxygenase (CAO), pheophorbide *a* oxygenase (PAO), 7-hydroxymethyl chlorophyll *a* reductase (HCAR) (for review, see [24]), and translocon at the inner chloroplast envelope 55 (TIC55) [25]. [Fe–S] clusters, together with siroheme (i.e., a specialized heme-like prosthetic group), are cofactors of sulfite and nitrite reductases (SiR and NiR) [26,27], both of which require reduced Fd as the physiological electron donor.

Mn

Mn is essential for light-driven oxidation of H_2O to extract the electrons needed in the photosynthetic electron chain and to release protons to generate the universal energy unit adenosine triphosphate (ATP). As a byproduct, O_2 is produced. For this purpose, four Mn ions constitute the catalytic center in the Mn_4CaO_5 cluster of the oxygen-evolving complex in PSII (Table 1 and Figure 1). In accordance with the Kok cycle [28], the Mn ions cycle through different oxidation states (Mn^{3+}, Mn^{4+}), the so-called S states, driven by the successive absorption of photons to extract electrons from H_2O [29]. PSII activity is therefore highly sensitive to Mn deficiency [30]. Moreover, a binuclear Mn^{2+} center is involved in recognition of the thylakoid-associated phosphatase 38 (TAP38) and phosphorylated light-harvesting chlorophyll *a/b* binding protein 1 (Lhcb1) during state transition; a mechanism allowing plants to regulate energy distribution between PSI and PSII [31,32].

Figure 1. (A) Iron (Fe) transport: At the outer envelope (OE) membrane, Fe uptake predominantly occurs as Fe-citrate. In the intermembrane space, ferric reductase oxidase 7 (FRO7) facilitates Fe reduction. Subsequently, Fe^{2+} is transported across the inner envelope membrane (IE) by the NiCo–PIC1 (nickel/cobalt transporter–permease in chloroplasts 1) transport system into the stroma, where it is provided for heme biogenesis and Fe–Sulfur [Fe–S] cluster assembly together with other Fe requiring enzymes (FeRE) or stored in ferritins when in excess. Frataxin (FH) delivers Fe to the [Fe–S] cluster assembly machinery, and [Fe–S] clusters are transferred to their target photosynthetic apoproteins via specific transfer proteins (TP), whereas the transport mechanisms for heme incorporation into photosystem II (PSII) and cytochrome (Cyt) b_6/f are unknown (grey arrows). Stromal Fe export is facilitated by the yellow stripe1-like (YSL)4/6 proteins. (B) Manganese (Mn) transport: The chloroplast Mn transporter 1 (CMT1) at the IE allows Mn uptake into the chloroplast stroma, where Mn^{2+} is required for activation of Mn responsive enzymes (MnRE). Stromal Mn^{2+} is imported into the thylakoid lumen via photosynthesis affected mutant 71 (PAM71), where it is assembled into the Mn_4CaO_5 cluster constituting the oxygen-evolving complex of PSII. The luminal protein PsbP possibly acts as a chaperone for Mn. (C) Copper (Cu) transport: The P-type ATPases, heavy metal ATPase (HMA)6 and HMA8, work in tandem for Cu^+ uptake at the IE and thylakoid membrane, respectively [40]. Furthermore, HMA1 functions in Cu uptake [and possibly export, depending on the actual situation (grey arrow)] at the IE. The plant-specific Cu chaperone plastid chaperone 1 (PCH1) provides the guided Cu^+ delivery to HMA6. The Cu chaperone for Cu/Zinc superoxide dismutase (Cu/ZnSOD) – CCS – is responsible for Cu delivery to Cu/ZnSOD and may also facilitate Cu delivery to HMA8 (grey arrow). The plastocyanin (PC) apoprotein is imported via the secretion system (Sec) pathway into the thylakoid lumen, where the protein is matured upon Cu incorporation. Abbreviations: Fd, ferredoxin; PetC, Rieske [2Fe–2S] protein; PSI, photosystem I; Tat, twin arginine translocase.

In the chloroplast stroma, enzymes of amino acid biosynthetic pathways are activated by Mn^{2+}. The imidazoleglycerol-phosphate dehydratase (IGPD) is a key enzyme in histidine biosynthesis [33–35]. The shikimate pathway of aromatic amino acid biosynthesis involves the Mn^{2+}-dependent 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHP) enzyme [36]. Recently, a disputable hypothesis has been put forward, that Mn-binding to Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) favors its oxygenation activity [37]; by doing so, the oxygenase reaction of Rubisco would generate additional reductants, which participate in the assimilation of nitrate into amino acids to mitigate carbon/nitrogen imbalances derived from atmospheric CO_2 fluctuations.

Cu

Plastocyanin (PC) is the small soluble Cu protein in the thylakoid lumen, mediating the electron transfer from Cyt b_6f to PSI (Table 1 and Figure 1). In arabidopsis, two PC isoforms are encoded that have an equivalent essential function in electron transport activity. However, the PC1 isoform accumulates at lower levels than PC2, which is more sensitive to Cu availability at the protein level [38,39]. Another abundant Cu enzyme, polyphenol oxidase (PPO), contains two Cu^{2+}/Cu^{+} ions per monomer. In the thylakoid lumen of spinach (*Spinacia oleracea*), the enzyme is thought to function in defense against biotic attacks [40]. However, genes encoding PPO are absent in arabidopsis and the enzyme is therefore likely dispensable for the primary metabolism [41,42].

In the stroma compartment, the major Cu enzyme is the ROS-detoxifying Cu/Zinc superoxide dismutase (Cu/ZnSOD), where Cu^{2+}/Cu^{+} acts as the redox active cofactor. Despite their colocalization in chloroplasts, FeSOD and Cu/ZnSOD share minimal structural homology and have evolved independently of one another [22,43]. The expression profiles of *SOD* genes in a number of plants indicate that both Cu/ZnSOD and FeSOD are even redundant under various conditions, such as salt, drought, and heat stress [44–46].

Chloroplast Responses to Environmental Transition Metal Deficiency

Transition metal deficiency manifests itself in reduced biomass production and a general discoloration of leaves, referred to as chlorosis. Cu deficient plants develop chlorotic symptoms that appear at the tip of the youngest leaves [47]. Fe and Mn deficiency cause interveinal chlorosis, which appears as a sharp distinction between veins and chlorotic areas of the youngest leaves [48,49].

At the cellular level, transition metal deficiency conditions often trigger disorganization of the chloroplast and its thylakoid membrane system. For instance, arabidopsis permease in chloroplasts 1 (*pic1*) mutants are unable to import Fe into chloroplasts, and their chloroplast number and size are reduced. Chloroplast development was found to be distorted with inchoate or even absent thylakoids [50]. Likewise, in Mn-deficient chloroplasts of arabidopsis chloroplast Mn transporter 1 (*cmt1*) mutants, chloroplast development was abnormal and thylakoids appeared disorganized, with either hypo- or hyper-stacked grana lamellae [15,16]. Clearly, Fe and Mn deficiencies directly affect the biogenesis of photosynthetic membrane complexes.

At the molecular level, genes associated with photosynthesis and tetrapyrrole metabolism are extensively downregulated under Fe deficiency [51]. Accordingly, impairment of chlorophyll biosynthesis is the most prominent sign of Fe deficiency. Decreased mRNA levels are also reflected in reduced protein levels of abundant Fe proteins, such as Fd (especially Fd2), proteins of the Cyt b_6f complex, and enzymes of the S assimilation pathway [52]. Reduced accumulation of proteins of the [Fe–S] cluster assembly system, S mobilization A and B (SufA and SufB), presumably avoids the risk of forming incomplete [Fe–S] clusters, which are likely to accelerate the formation of the highly reactive hydroxyl radical in the Fenton reaction [52]. For the apoprotein SufA, the lack

of Fe as cofactor may cause protein instability rather than decreased mRNA levels [52]. Disrupting the transfer of [Fe–S] clusters to their target proteins, such as Fd, triggers leaf Fe-deficiency responses and, interestingly, results in overaccumulation of Fe in the chloroplast [53].

In the absence of Mn, the PSII supercomplexes are less stable [54,55]. A conceptual model explains the main events of Mn deficiency in PSII with Mn deficiency leading to reduced Mn binding in PSII complexes, which causes disintegration of PSII supercomplexes [56]. Consequently, the PSII core is increasingly affected by oxidative damage, which lowers PSII yield and eventually CO_2 assimilation and biomass production [30,54]. To prevent those detrimental effects, plants are able to mobilize stored Mn from the vacuole and recruit it to the chloroplast [57]. Recently, a group of ancient barley landraces was demonstrated to have a superior Mn efficiency trait, which enables the landraces to maintain Mn binding in PSII under Mn-deficient conditions [49].

Cu deficiency does not alter the expression level of the *PC* genes but instead triggers a large molecular remodeling that allows Cu to be preferentially allocated to PC [58–60]. In order to achieve this, transcripts of *Cu/ZnSOD* undergo microRNA-mediated downregulation. As a result, Cu provision is prioritized toward PC in plant chloroplasts [2,40,58,60,61].

Chloroplasts Membranes – Barriers to Transition Metal Import and Mobilization

Due to their endosymbiotic origin, chloroplasts are surrounded by a double-membrane envelope, and thus Fe, Mn, and Cu have to pass the outer envelope (OE) and the inner envelope (IE) (Figure 1). In addition, the thylakoid membrane system comprises the chloroplast internal extensive membrane system, and at least Mn^{2+} and Cu^{2+} need to cross this membrane to reach their destinations in the lumen (Figure 1).

Chloroplast Fe Transport

The uptake of Fe into chloroplasts is relatively well studied (Figure 1A). Studies in sugar beet (*Beta vulgaris*) [62] and oilseed rape (*Brassica napus* L.) [63] have demonstrated that Fe^{3+}-citrate is the preferred substrate for chloroplast Fe uptake across the OE. Once in the chloroplast intermembrane space, Fe transport across the IE occurs most likely in the form of Fe^{2+} [64,65]. In arabidopsis, it involves the ferric reductase oxidase 7 (FRO7) [66] facilitating Fe^{3+} reduction for the subsequent transport of Fe^{2+} across the IE. In sugar beet, the activity of the Fe^{3+} reductase enzyme was clearly associated with the IE and required the operation of the photosynthetic electron transport chain for providing reducing power [67].

PIC1 provided the first evidence for Fe^{2+} transport across the chloroplast IE membrane [50,64,68]. PIC1 is proposed to be part of a larger Fe import complex also involving the putative metal transport protein NiCo, which belongs to the Ni^{2+}-Co^{2+} transporter family (TC#2.A.52) according to the Transporter Classification Database [69]. The current model is that Fe is first bound by NiCo and subsequently transferred to PIC1. The mode of Fe^{2+} uptake employs the proton motif force across the IE [64,68]. The strong coexpression of *PIC1*, *NiCo*, and *FRO7* observed in oilseed rape [63] supports the reduction-based Fe transport system of chloroplasts. An alternative pathway to PIC1-dependent Fe uptake has been suggested to involve the multiple antibioticresistance1 (MAR1) and the mitoferrin-like 1 (MFL1) proteins, although their integration into the IE have not been unequivocally shown. MFL1 belongs to the mitochondrial carrier family proteins but is annotated as an IE chloroplast protein [9]. It has a predicted chloroplast targeting peptide and might be involved in Fe transport under Fe excess [70]. Similarly, the MAR1 protein has been speculated to act as transporter for Fe chelators, such as citrate or nicotianamine [71]. However, the expression of *MAR1* in oilseed rape was much lower than the *FRO7-PIC1-NiCo* system under Fe-replete conditions [63] and is downregulated by 60% under Fe deficiency

[71], suggesting a minor importance of this transporter in Fe delivery under these conditions. Recently, a prokaryotic-type ATP-binding cassette (ABC) transporter module has been implicated in metal uptake at the IE membrane [72]. This module consists of the membrane intrinsic ABC subfamily I 12 (ABCI12) and the soluble ABCI10 component, which is the nucleotide-binding domain. A third player, ABCI11, also known as NAP14 (for nonintrinsic ABC protein 14), might be attached to the IE and/or to plastoglobuli [72,73]. Analysis of *abci10* and *abci11* (=*nap14*) showed severe growth defects and strong chlorosis accompanied by impaired transition metal homeostasis in both mutants [72,73]. Whereas ABCI11 (NAP14) is involved in Fe homeostasis [73], the transport specificity of the ABC10/ABC12 module at the IE awaits the molecular identification of the substrate binding component(s).

To regulate chloroplast Fe concentration, the two yellow stripe1-like (YSL) proteins YSL4 and YSL6, are hypothesized to function in Fe efflux from the chloroplast to the cytoplasm. The envelope localization of the proteins is still under debate, but the expression of the genes is upregulated in response to Fe excess [74]. In *Zea mays*, the Fe deficiency-related 4 (ZmFDR4) protein, a probable thylakoid membrane-localized protein, is more strongly expressed under Fe deficient than under Fe replete conditions [75]. Thus, FDR4 could be involved in Fe uptake across the thylakoid membrane in monocots.

Chloroplast Mn Transport

Despite the fundamental need for Mn in oxygenic photosynthesis, chloroplast Mn transporters were only recently identified (Figure 1B). The IE CMT1 [15,16] and the thylakoid photosynthesis affected mutant 71 (PAM71) protein [54], synonymous to putative chloroplast calcium (Ca) proton antiporter (CCHA) [76], act in tandem to efficiently deliver Mn to the thylakoid lumen for its incorporation into the Mn_4CaO_5 cluster in PSII [15,77]. CMT1 and PAM71 belong to the unknown protein family UPF0016 [78]. This protein family was recently established as functioning in transport processes. Loss of function *cmt1* arabidopsis mutants show strongly reduced growth accompanied by chloroplasts having aberrant thylakoid membrane systems and diminished integral photosynthesis protein complexes due to strongly reduced chloroplast Mn content. Plant growth can hardly be complemented by supplementation of excess Mn concentrations in the growth medium, and CMT1 is therefore likely to be the dominant Mn uptake system at the IE of chloroplasts [15,16]. The p*am71* mutant grows comparably better than the *cmt1* mutant and is specifically impaired in PSII activity. This is caused by reduced Mn incorporation into PSII supercomplexes [54]. Notably, loss of PAM71 in both arabidopsis and the green alga *Chlamydomonas reinhardtii* can be compensated by enhanced Mn additions, indicating the presence of an uncharacterized low-affinity uptake system for Mn at the thylakoid membrane [54,78].

For the CMT1 and PAM71 proteins, an additional role in Ca transport has been suggested, and the proteins were named bivalent cation transporter (BICAT) 1 and 2, respectively [79]. BICAT1/PAM71 is proposed to transport Ca^{2+} into the thylakoid lumen and BICAT2/CMT1 to transport Ca^{2+} across the chloroplast IE [79]. However, Ca contents do not differ between *cmt1* mutant chloroplasts and wild-type (WT) chloroplasts [16], and *pam71* mutants even accumulate more Ca in the thylakoid lumen with respect to the WT [54]. The physiological effects in cyanobacteria mutants of the orthologous gene [80,81] underline the main function of the UPF0016 family is Mn transport in oxygenic photosynthetic organisms.

Chloroplast Cu Transport

The chloroplast Cu uptake and intraorganellar distribution is facilitated by two P_{IB}-type ATPases, P-type ATPase of arabidopsis 1 and 2 (PAA1 and PAA2), also known as heavy

metal ATPase 6 and 8 (HMA6 and HMA8), which function in tandem in the transport of monovalent Cu^+ ions across the chloroplast envelope and the thylakoid membrane, respectively [13,82,83] (Figure 1C). Biochemical analyses have revealed that both proteins are high-affinity Cu^+ transporters but differ in their biochemical properties as HMA8 has a higher affinity for Cu^+ than HMA6 [84]. Cu excess in the stroma triggers HMA8 degradation [59]. The phenotypes of the arabidopsis mutants *hma6* and *hma8* specifically relate to reduced Cu delivery to the major plastid Cu sinks. As expected, the *hma6* mutant shows impaired stromal Cu/ZnSOD activity, and both *hma6* and *hma8* mutants have a strong reduction in holo-PC protein content, resulting in impaired photosynthetic electron transport [82]. An additional member of the HMA family, the broad-specificity HMA1 protein, is suggested to facilitate ATP-dependent Cu import at the chloroplast IE. This pathway is distinct from HMA6 and becomes vital only under high-light conditions [85,86]. Besides Cu import into arabidopsis chloroplasts, Cu (and Zn) export activity has been reported for HvHMA1 in barley chloroplasts for remobilization purposes [87,88].

Transport Pathways and Mechanisms Ensuring Correct Protein Metalation in Chloroplasts

Chloroplast metalloproteins functioning in photosynthetic electron transport are assumed to obtain their metal from exchangeable stromal pools and are subject to the universal order of protein divalent metal-binding affinities described by Irving and Williams [3], with Cu forming tighter complexes than Fe and Mn, respectively. This challenges the use of transition metals in photosynthesis to ensure correct metal acquisition by apometalloproteins but can be overcome by either compartmentalized protein folding or by assistance of metal delivery proteins, known as metallochaperones. Metallochaperones pass metals between transporters and their target apoproteins via ligand-exchange reactions. Thus, they participate in the final transport step before crossing the thylakoid membrane, which is mediated by one of two distinct pathways; the chloroplast secretion system (Sec) or the chloroplast twin arginine translocase (Tat) pathway [89]. The translocon of the Sec-type pathway only accepts unfolded proteins, whereas the Tat-type pathway accommodates transport of mature and highly folded proteins [90].

Fe Metalation

Assembly of [Fe–S] clusters needed in stroma-localized proteins and proteins of the electron transport chain takes place in the stroma compartment by the SUF pathway. In the first stage, [Fe–S] clusters are built from Fe and S delivered by proteins onto so-called scaffold proteins, which are the primary sites of *de novo* cluster assembly [24]. However, the source of Fe and how Fe is delivered to those scaffold proteins are unknown. The Fe-storage protein ferritin (FER) was considered a candidate; however, the triple *fer1fer3fer4* arabidopsis mutant of three major FERs retained fully functional photosynthetic apparatus, suggesting that FERs are not the major Fe pool for the SUF pathway [91]. Instead, current models assume that a metallochaperone acquires Fe and directly donates Fe to the assembly pathway by interacting with one of the scaffold proteins. Frataxin (FH) is a metallochaperone candidate for [Fe–S] cluster assembly [92,93] (Figure 1). After assembly, [Fe–S] clusters are transferred to their respective target recipient apoprotein with the help of transfer proteins. A total of nine potential chloroplastic [Fe–S] transfer proteins have been identified so far [24]. The unidirectional and intact transfer of [Fe–S] clusters from one transfer protein to another indicates that some [Fe–S] transfer proteins work in sequence and most possess specificity to individual [Fe–S] cluster types. For example, [4Fe–4S] of PSI proteins are supplied by the chloroplast-localized nifU-like proteins NFU2 and NFU3, and high-chlorophyll fluorescence 101 (HCF101) [24,94,95]. The Asn-Glu-Glu-Thr motif protein NEET is proposed to transfer [2Fe–2S] clusters to Fd [53]. It remains elusive which transfer

protein delivers the [2Fe–2S] cluster to PetC (Table 1) prior to translocation across the thylakoid membrane via the Tat pathway to reach its destination on the lumenal side of the Cyt b_6/f complex [96] (Figure 1).

Mn Metalation

The three lumenal proteins PsbO, PsbP, and PsbQ of PSII are known to protect and stabilize the oxygen-evolving complex and maintain optimal Ca and chloride concentrations in plant chloroplasts [97]. The crystal structure of PsbP from spinach was shown to contain two specific Mn^{2+}-binding sites, with one site having a relatively low affinity [98], making it a suitable candidate site for Mn donation to the Mn_4CaO_5 cluster assembly. Thus, a role for PsbP as a metallochaperone for Mn^{2+} in the thylakoid lumen has been suggested [56,98–100]. In line with this assumption, the ancestral form of PsbP in cyanobacteria, CyanoP, has been proposed as a promising Mn chaperone. Experimental support derives from the observation that the *Synechocystis* Δ*SynPAM71* mutant overaccumulates CyanoP, maybe to buffer excess amounts of Mn in this strain [81].

Cu Metalation

The plant-specific Cu chaperone plastid chaperone 1 (PCH1), generated by an alternative splicing event of *HMA6* pre-mRNA, resides in the intermembrane space. PCH1 contains an N-terminal metal-binding domain, which enables transient interaction of PCH1 to the intramembrane metal-binding site of HMA6 for Cu delivery across the IE [40,61,101]. In the stroma, another Cu chaperone, Cu chaperone for Cu/ZnSOD (CCS), is responsible for Cu delivery to Cu/ZnSOD [102]. CCS was further identified as a possible source for the targeted Cu provision to the thylakoid transporter HMA8. Thus, CCS might be involved in Cu delivery to PC [101] (Figure 1). Even so, the CCS knockout mutant did not show alterations in PSII activity, nor changed PSI and PSII protein amounts, even after high-light stress, when grown on high Cu media [102]. These findings imply the existence of further Cu metallochaperones in the chloroplast stroma. PC is a Sec substrate, and thus Cu insertion and PC maturation must be completed within the thylakoid lumen, suggesting that Cu chaperones are also required in this compartment.

Concluding Remarks and Future Perspectives

Chloroplasts respond instantaneously to changes in transition metal concentrations to regulate the mechanisms controlling transition metal transport and storage in the cell. Despite recent progress in identification and characterization of chloroplast transition metal transporters, major questions await answering, including the exact transport mode, the involvement of chaperones, and the nature of their substrate state. For the proteins involved, the unequivocal determination of their subcellular localization is essential. This is of special importance as accumulating evidence points towards dual organelle functions (in, e.g., chloroplast and mitochondrion/vacuole/endoplasmic reticulum) for a subset of transport proteins involved.

Although fundamental, little is known about chloroplast metal sensing (Box 1) and how metalloproteins can discriminate and acquire the correct metal cofactor in the complex matrix of the intermembrane space, stroma, and lumen fractions. This adds an additional layer of complexity to chloroplast transition metal regulation. Future work should integrate molecular metal sensors and omics approaches to gain a comprehensive knowledge of the sophisticated networks of response towards depletion and repletion of transition metal ions. This will provide new knowledge of chloroplast transition metal regulation in response to fluctuating transition metal availability and will contribute to our understanding and ultimate engineering of plant photosynthetic efficiency and productivity (see Outstanding Questions).

Outstanding Questions

How is the demand of the individual transition metals coordinated?

How is sequestration and remobilization of transition metals into/from storage pools (e.g., the vacuole) regulated?

How are heme cofactors assembled into photosynthetic proteins?

What is the transport mode of the UPF0016 family proteins CMT1 and PAM71 driving Mn transport?

Are Mn chaperones present in the chloroplast stroma and thylakoid lumen?

What is the molecular nature of the uncharacterized low-affinity Mn uptake systems at the thylakoid membrane and/or chloroplast envelope membrane? Do these low affinity systems also accept other transition metals, such as Cu and Fe?

Does a further stromal Cu chaperone exist for the thylakoid membrane-localized HMA8 transporter?

Are there specific ligands for Mn, Fe, or Cu in the chloroplast or are ligands similar to metal–ligand complexes in phloem and xylem sap?

Box 1. Chloroplast Metal Sensing and Regulation

When exposed to either metal deficiency or excess, the chloroplast needs to react instantaneously to avoid harmful imbalances affecting the photosynthetic electron transfer chain. Despite the central importance of maintaining photosynthetic efficiency, the knowledge on metal orchestration, metal interactions, and signaling within the chloroplast is surprisingly poor.

The main questions of chloroplast transition metal regulation are how metal concentrations and individual ratios are sensed within the chloroplast and how the specific metal concentration is eventually controlled. Impaired electron transfer, owing to imbalanced availability of metalloproteins of the photosynthesis apparatus, leads to generation of overreduction signaling molecules, such as ROS [15]. It is well known that information via ROS serves to tune processes inside and outside the chloroplast [114,115]. Metal homeostasis strategies, such as metal chelation and transport, are under nuclear transcriptional control and induced upon transition metal deficiency or excess [40,51,116]. Although speculative, the chloroplast may act as a cellular sensing and signaling site for metal deficiencies, and ROS production might induce a signaling cascade, regulating the expression of genes coding for metal importers (or exporters) at the IE. The analysis of the *cmt1* mutant supports the central role of metal transporters at the IE in chloroplast metal regulation; loss of the Mn transporter CMT1 results in strongly reduced Mn concentrations in the chloroplast leading to reduced PSII activity [15,16]. To accommodate deficiencies in PSII, adjustment of photosystem stoichiometry is achieved by decreasing subunits of PSI and Cyt b_6/f. Hence, surplus Fe has to be sequestered. Possibly, excess Fe is exported from the chloroplast and sequestered into the vacuole, the central cellular hub for transition metals. Thus, low Mn concentration implicates reduced levels of chloroplast Fe, yet Cu concentrations are unaffected [15].

Not only that, the chloroplast sends signals to the nucleus (retrograde signaling), cytosolic metal sensing triggers anterograde pathway (e.g., when plants experience Mn stress, expression of *CMT1* is downregulated) [15], and excess cytosolic Fe triggers *FER* expression [116]. Chloroplast transition metal requirement is dynamically changing and thus requires not only communication between this organelle and the nucleus but also long-distance signaling between the shoot and the root [117] as primary source for transition metal acquisition.

Acknowledgments

This research was financially supported by the Independent Research Fund Denmark – Technology and Production Sciences (grant DFF-5054-00042 to S.B.S) and the German Research Foundation (Deutsche Forschungsgemeinschaft) – EI945/3-1 to M.E. and SCHN560/4-1 to A.S. The authors wish to thank Debbie Maizels, Zoobotanica Scientific Illustration, for providing illustrations for Figure 1.

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